REMARKS

The status of the claims is as follows:

Original:	2 and 5
Currently amended:	1, 3, 4, 6, 7-10, 13 and 16
Previously presented:	12
Canceled:	11, 14, 15 and 17
Withdrawn:	None
New:	18-21

Claims 1-10, 12, 13, 16 and 18-21 will be pending with entry of this amendment. Reconsideration is requested.

The title of the invention has been amended on page 1. The amended title is the same as the title suggested by the Examiner, except that the phrase "AND RELATED COMPOUNDS" is not included.

Claims 15 and 17 were canceled in the amendment filed June 22, 2005. Claims 11 and 14 have been canceled herein. Applicants hereby reserve the right to pursue the subject matter set forth in the canceled claims in one or more continuing applications.

Claims 18-21 are new, each reciting a particular embodiment of the invention.

The other claim amendments are discussed below.

None of the amendments made herein introduces new matter.

Information Disclosure Statements

Receipt of initialed copies of the 1449 forms from the information disclosure statements filed 4/24/2006 and 4/12/2007 is acknowledged.

Claim objection

Claim 10 has been objected to due to a missing parenthesis in the 79th species. The 79th species has been removed from claim 10 as non-elected subject matter. Withdrawal of the objection is requested.

Improper Markush rejection

Claims 1-14 and 16 have been rejected as drawn to an improper Markush group. Per the Examiner's suggestion, the non-elected subject matter has been removed from the claims.

Applicants reserve the right to pursue the removed subject matter in one or more continuing applications. Withdrawal of this rejection is requested.

Rejections under 35 U.S.C. § 112, second paragraph

(i) Claims 1, 2, 12-14 and 16 have been rejected under 35 U.S.C. § 112, second paragraph, as being indefinite. The Examiner has asserted that the phrases "such as" and "e.g., ..." render the claims indefinite. The parenthetical expressions directed to "e.g., ..." in the definition of R^4 in claim 1 have been removed. No occurrence of "such as" was found in any of the claims. Withdrawal of this rejection is requested.

(ii) Claims 3 and 5 have been rejected under 35 U.S.C. § 112, second paragraph, as being indefinite. The Examiner has asserted that the definition of R^4 has insufficient antecedent basis. Claim 3 has been rewritten in independent form, and claim 5 indirectly depends from claim 3. Withdrawal of the rejection is requested.

(iii) Claims 4 and 6 have been rejected under 35 U.S.C. § 112, second paragraph, as being indefinite. The Examiner has asserted that the definition of R^4 has insufficient antecedent basis. In view of the amendment to claim 3 described in part (ii) above, withdrawal of this rejection is requested.

(iv) Claims 1, 2, 12-14 and 16 have been rejected under 35 U.S.C. § 112, second paragraph, as being indefinite. The Examiner has asserted that the substituent "oxo" in the definition of HetA is indefinite. As would be understood by the person of ordinary skill in the art, an "oxo" substituent is possible on a heteroaromatic ring as a result of keto-enol tautomerization. The "oxo" has been replaced with "OH" to make the definition more clear. Withdrawal of this rejection is requested.

(v) Claim 13 has been rejected as indefinite. The Examiner has asserted that the terms "HIV protease inhibitors", "non-nucleoside HIV reverse transcriptases" and "nucleoside HIV reverse transcriptase inhibitors" are indefinite. This rejection is traversed. Each of these terms was well understood as of the time the subject application was filed. In vitro assays have been available for many years to ascertain whether or not a particular substance is or is not an active inhibitor of HIV protease or HIV reverse transcriptase. See, e.g., the assay in col. 58 of US5413999 (protease) and the assay in cols. 30-31 in US5519021. Other in vitro assays have been available to measure the activity of such compounds in the inhibition of HIV replication. See, e.g., Vacca et al., *Proc. Natl. Acad. Sci. USA* 1994, 91: 4096 (cited on page 28, line 16 of the specification; copy enclosed). It was also known that compounds identified as HIV protease inhibitors (PIs) and HIV reverse transcriptase inhibitors (RTIs) were useful clinically for the treatment of HIV infection; e.g., PIs - indinavir, ritonavir, nelfinavir, and saquinavir; non-nucleoside RTIs - zidovudine, lamivudine, and abacavir; nucleoside RTIs - nevirapine,

delavirdine, and efavirenz. With this background knowledge, the person of ordinary skill in the art would have a clear understanding of what these terms refer to and how to determine with a reasonable degree of certainty what substances lie within their scope. Withdrawal of this rejection is requested.

Rejection under 35 U.S.C. § 112, first paragraph

Claims 13 and 16 have been rejected under 35 U.S.C. § 112, first paragraph, as not enabled for prevention. Per the Examiner's suggestion, the references to prevention and delay in the onset have been removed from claims 13 and 16. Withdrawal of this rejection is requested.

The application is believed to be in condition for allowance and passage to issue is requested. The Examiner is invited to telephone the undersigned should any minor matters need to be resolved before a Notice of Allowance can be mailed.

Respectfully submitted,

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L-735,524: An orally bioavailable human immunodeficiency virus type 1 protease inhibitor

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ABSTRACT **To date, numerous inhibitors of the human immunodeficiency virus type 1 protease have been reported, but few have been studied extensively in humans, primarily as a consequence of poor oral bioavailability in animal models. L-735,524 represents a class of human immunodeficiency virus type 1 protease inhibitors, termed hydroxyaminopentane amides, that incorporate a basic amine into the hydroxyethylene inhibitor backbone. L-735,524 is a potent inhibitor of virus replication in cell culture and inhibits the protease-mediated cleavage of the viral precursor polyproteins that results in the production of noninfectious progeny viral particles. The compound is effective against viruses resistant to reverse transcriptase inhibitors and is synergistically active when used in combination with reverse transcriptase inhibitors. Most importantly, L-735,524 exhibits good oral bioavailability and plasma pharmacokinetic profiles in two species of laboratory animals by using clinically acceptable formulations. Accordingly, the compound was selected for evaluation of safety and pharmacokinetic studies in humans.**

The human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of AIDS. The viral genome encodes a 99-amino acid protease that, as a homodimer, mediates the cleavage of the viral precursor polyproteins (1–6). The cleavages are required for the production of infectious progeny virions as the genetic inactivation of the enzyme results in the elaboration of noninfectious viral particles (6). Accordingly, HIV protease inhibitors offer an attractive strategy for arresting the progression of HIV infection and its pathogenic sequelae (7, 8).

A number of highly potent HIV protease inhibitors based upon the transition-state mimic concept have been reported (9, 10), and many display potent antiviral activity in cell culture. However, most of these peptidomimetic compounds have not been studied extensively in humans primarily as a result of poor aqueous solubility and inadequate oral bioavailability in animal models. We now report an example of a class of HIV-1 protease inhibitors designated hydroxyaminopentane amide compounds (11). This series, exemplified by L-735,524 (Fig. 1, compound 5), incorporates a basic amine into the backbone of hydroxyethylene transition-state mimic compounds, such as compounds 2 and 3 (12–14), which contain (1*S*)-amino-(2*R*)-hydroxyindan as a C-terminal amino acid surrogate. Some inhibitors from this hydroxyaminopentane amide class exhibit good aqueous solubility and show enhanced oral bioavailability in animals when administered in clinically acceptable formulations. This report describes the properties of L-735,524 whose *in vitro* antiviral activity and animal bioavailability supported its selection for clinical evaluation.

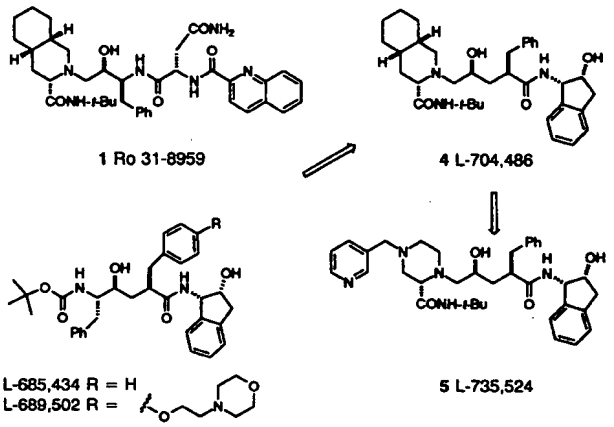

FIG. 1. Structure of L-735,524. *t*-Bu, tertiary butyl; Ph, phenyl.

MATERIALS AND METHODS

Preparation of L-735,524 and Analogs. The synthesis of L-735,524 and analogs is described in detail elsewhere (15). The compound is a white crystalline powder isolated as a monohydrate that melts at 167.5–168°C. Its solubility in water at pH 7.0 is 0.015 mg/ml, increasing at pH 4.0 to >1.5 mg/ml.

Protease Inhibition Assays. Assay of HIV-1 and HIV type 2 (HIV-2) protease inhibition was performed by peptide cleavage using the substrate Val-Ser-Gln-Asn-β-naphthylalanine*Pro-Ile-Val. Reactions were carried out at pH 5.5 (50 mM sodium acetate/0.1% bovine serum albumin) for various times and were terminated with 5% (wt/vol) H_3PO_4 (16). Products of the cleavage reaction were quantified by HPLC detection, and the data were fit to nonlinear least squares equations for determination of steady-state Michaelis–Menten kinetics.

Other proteolytic enzymes also were tested for inhibition (see *Results*). Two assays were performed for each enzyme. These included a peptide cleavage and hydrolysis assay (17). Human plasma renin was assayed by the Incstar (Stillwater, MN) test kit.

Acute Infection Assay. The assay was performed using MT-4 human T-lymphoid cells, human peripheral blood mononuclear cells (PBMCs), or primary human monocytes/

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Abbreviations: HIV, human immunodeficiency virus; RT, reverse transcriptase; PBMC, peripheral blood mononuclear cell; DMSO, dimethyl sulfoxide; AZT, 3'-azido-3'-deoxythymidine.

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macrophages. MT-4 cells (2.5×10^5 cells per ml) were infected at a multiplicity of infection of ≤0.01 and incubated overnight in RPMI 1640 culture medium containing 10% (vol/vol) fetal bovine serum. The infected cells were then washed twice in fresh culture medium and seeded into 96-well cell culture plates at 5.0×10^4 cells per well. Serial 1:2 dilutions of the test compounds were added to the wells. The test cultures were incubated for an additional 72 h at 37°C at which time virus levels were assayed.

Uninfected PBMCs were obtained from the whole blood of human donor volunteers by Ficoll gradient separation. After washing, the cells were resuspended at 2.5×10^5 cells per ml in RPMI 1640 cell culture medium containing 10% fetal bovine serum and phytohemagglutinin at 5.0 μg/ml. After 5 days of incubation, the activated cells were resuspended in phytohemagglutinin-free culture medium with interleukin 2 at 30 units/ml. The cells were incubated for an additional 3 days prior to use. The assay was performed by infecting 5.0×10^5 cells per ml at a multiplicity of infection of <0.01. After 24 h of incubation, the cells were washed, resuspended in fresh medium, and seeded into 96-well cell culture plates at 2.0×10^5 cells per well. Serial 1:2 dilutions of the test compound were then added to the wells. The cultures were incubated at 37°C and virus growth was assessed at 4–6 days after infection.

Primary human monocytes/macrophages were obtained by placing fresh PBMCs in gelatin-coated cell culture flasks. After a 2-h incubation, the adhered cells were washed free of nonadherent cells. The attached monocytes were removed with EDTA, washed, and added to 96-well cell culture plates at 5.0×10^5 cells per well in Dulbecco's modified Eagle's medium with 10% (vol/vol) human AB serum, 10% (vol/vol) horse serum, granulocyte/macrophage colony-stimulating factor at 1000 units/ml, and macrophage colony-stimulating factor at 50 units/ml. The cultures were maintained for 7–10 days prior to infection with virus. After a 3-day viral adsorption period, the cells were washed and fed with fresh culture medium containing serial 1:2 dilutions of the test compound. Incubation was continued at 37°C and the cultures were fed every 48–72 h with medium and compound. Virus growth was assessed at 14–21 days after infection.

Virus levels in the test cultures were assayed using a commercial viral core p24 antigen assay kit (Coulter). The 95% cell culture inhibitory concentration (IC_{95}) was defined as the concentration of test compound that inhibited virus p24 antigen production by at least 95% relative to untreated control cultures.

Synergy. The antiviral activities of combinations of inhibitors were analyzed for possible synergism by the method of Elion *et al.* (18). Fractional inhibitory concentrations were calculated as described. Combination assays were performed using the HIV-1_{IIIb} variant and MT-4 T-lymphoid cells.

Viral Core Protein Processing. H9 human T-lymphoid cells, persistently infected with the HIV-1_{IIIb} variant, were washed with culture medium (RPMI 1640 medium/10% fetal bovine serum) and resuspended at 5.0×10^4 cells per ml in fresh medium containing the appropriate concentration of L-735,524. After 24 h at 37°C, the cells were harvested, washed, and resuspended in medium containing the inhibitor. After an additional 24 h of incubation, the cell-culture supernatant was harvested and concentrated 40-fold. The viral particles were then solubilized in buffer containing SDS and analyzed by SDS/PAGE. Viral proteins in the gel were visualized by Western blot analysis using an anti-HIV-1 human antiserum.

Mutant Protease Inhibition and Activity. Mutant HIV-1 protease was constructed, expressed in recombinant bacteria, and purified as described by Sardana *et al.* (19). Protease activity was determined by HPLC peptidolytic assay as described above. K_m values were calculated by fitting the velocity vs. substrate concentration to the Michaelis–Menten equation (hyperbolic) using KALEIDAGRAPH (Synergy Software, Reading, PA). The velocities were determined at eight substrate levels (50–1000 μM). The initial velocity and steady-state conditions for the enzymic reaction with each mutant enzyme were determined. k_{cat} values were calculated from V_{max} values by assuming 100% activity of the added enzyme and one active site per 22 kDa of dimeric protease. There was no further dissociation of the protease dimer into its monomeric (inactive) form during the course of the assays for all the mutant and wild-type enzyme kinetic analysis. For K_i determinations, the assay was performed at substrate concentrations ranging from 220 to 1000 μM with at least eight inhibitor concentrations. L-735,524 was initially dissolved in dimethyl sulfoxide (DMSO) and dilutions in DMSO were performed to give a final concentration of 2% DMSO in each assay sample. The K_i values were calculated using the equation $I_t/(1 - V_i/V_0) = E_t + K_i[(S + K_m)/K_m]V_0/V_i$ (20) (where I_t is inhibitor concentration, V_i is velocity at inhibitor concentration, V_0 is velocity in absence of inhibitor, E_t is total enzyme concentration, and S is substrate concentration) for tight binding inhibitors. The equation was solved with SIGMA PLOT (Jandel, San Rafael, CA). The enzyme concentrations were determined by amino acid analysis. In the K_i experiments, the enzyme concentrations used were as follows: wild type HIV-1, 0.06 nM; HIV-2, 0.67 nM; L23I, 0.15 nM; L23V, 0.26 nM; V32I, 0.47 nM; I47V, 0.16 nM; I47L, 1.9 nM; I50L, 0.19 nM; L76M, 0.10 nM; V82I, 0.04 nM; I84L, 0.14 nM; I84V, 0.32 nM.

Animal Pharmacokinetic Studies. Pharmacokinetic parameters of L-735,524 were studied in rats, dogs, and rhesus monkeys. In the rat intravenous (i.v.) studies, three male Sprague–Dawley rats (250–400 g) received the compound at 5.0 mg/kg i.v. in DMSO (1 ml/kg) via an implanted cannula. For oral studies, L-735,524 at 20.0 mg/kg was given by gavage as a solution in 0.05 M citric acid (5.0 ml/kg) or as a suspension in 0.5% methylcellulose (5.0 ml/kg) to each rat in a group of three rats after an overnight fast. The absolute oral bioavailability was determined by comparing the mean area under the curves obtained from each group of rats. The compound was also studied in male dogs (10–12 kg). After an overnight fast, each dog in a group of three animals received either an oral dose of compound (10.0 mg/kg) as a solution in 0.05 M citric acid (5.0 ml/kg) or as a suspension in 0.5% methylcellulose (5.0 ml/kg). The absolute oral bioavailability was determined by comparing the area under the curves after oral administration with that obtained after a subsequently administered i.v. dose of L-735,524 (2.0 mg/kg) in DMSO (0.1 ml/kg) to each of the dogs used in the oral experiment. An identical procedure was used to examine the pharmacokinetics and oral bioavailability in three *Cercopithecus* monkeys.

In all of the animal studies, blood samples were obtained after dosing at appropriate times, and plasma was obtained by immediate centrifugation and kept frozen (−20°C) until analyzed. L-735,524 plasma concentrations were determined by HPLC analysis after extraction of the compound with ethyl ether.

Plasma Protein Binding. Binding of L-735,524 to human, dog, and rat plasma was determined by an ultrafiltration method. L-735,524 was added to plasma to yield a final concentration range of 0.5–10.0 mg/ml. The compound solution was added in a volume equal to or less than 1.0% of the plasma volume. After incubation of plasma samples at 37°C for 5 min, 0.8 ml of plasma was immediately transferred to a Centri-free tube (Amicon). The tube was then centrifuged at $1500 \times g$ for 15 min at 37°C. The unbound fraction of the drug was estimated directly from the ratio of drug concentration in the ultrafiltrate to the total drug concentration in the original plasma samples before centrifugation as determined by HPLC.

RESULTS

***In Vitro* Anti-Protease Activity.** The structure of L-735,524 is shown in Fig. 1. The compound potently and competitively inhibits HIV-1 and HIV-2 proteases with K_i values of 0.34 and 3.3 nM, respectively. No significant inhibition of several other proteolytic enzymes was observed at L-735,524 concentrations exceeding 10 μM. These included the mechanistically related aspartyl proteases, human cathepsin D, porcine pepsin, bovine chymosin, and human plasma renin, and the serine proteases, factor Xa and elastase.

***In Vitro* Antiviral Activity.** L-735,524 was tested for its ability to inhibit virus spread in an acute infection assay. The results (Table 1) show that the compound was an effective inhibitor of both T-lymphoid-cell-adapted HIV-1 variants and primary virus isolates. Viral susceptibility to the protease inhibitor was not associated with susceptibility to either 3'-azido-3'-deoxythymidine (AZT) or a nonnucleoside reverse transcriptase (RT) inhibitor. L-735,524 was also effective in preventing acute infection spread of simian immunodeficiency virus (SIV_{mac251}) and of a monocytotropic HIV-1 variant.

L-735,524 was assayed for antiviral activity in combination with AZT, dideoxyinosine, or L-697,661, a nonnucleoside RT inhibitor. In all cases, the fractional inhibitory concentrations (18) were <1.0, suggesting that each combination was synergistic in its activity (data not shown).

Finally, cell viability assays demonstrated that the inhibitor was not toxic to MT-4 T-lymphoid cells at concentrations up to 400 μM (data not shown).

Inhibition of Viral Core Processing. The antiviral activity of HIV-1 protease inhibitors has been associated with the inhibitor-mediated prevention of virus core maturation that results from the incomplete processing of the viral core precursor protein (6). Accordingly, the ability of L-735,524 to prevent core protein cleavage was assessed using a persistently infected human T-lymphoid cell line. At inhibitor concentrations of 0.5 and 12.0 μM, the quantity of mature virion matrix (p17) and capsid (p24) proteins was substantially decreased relative to the amount present in untreated virions (Fig. 2). The relative quantity of the p55 core precursor and of the processing intermediates was increased. In addition, the inhibitor prevented the incorporation into viral particles of the mature RT subunits (p51 and p66) and of the mature viral integrase enzyme (p31). Both subunits are processed by the viral protease from a polyprotein precursor.

Table 1. Inhibition of acute virus infection by L-735,524 in cell culture

Virus	Cell type	IC_{95}, nM
$HIV\text{-}1_{IIIb}$	MT-4 lymphoid	100.0
$HIV\text{-}1_{MN}$	MT-4 lymphoid	50.0
$HIV\text{-}1_{RFII}$	MT-4 lymphoid	100.0
$HIV\text{-}1_{112}$	PBMC	100.0
$HIV\text{-}1_{139\text{-}8}$	PBMC	50.0
$HIV\text{-}1_{421}$	PBMC	25.0
$HIV\text{-}1_{5002}$	PBMC	100.0
$HIV\text{-}1_{5003}$	PBMC	100.0
$HIV\text{-}1_{5004}$	PBMC	100.0
$HIV\text{-}1_{SF162}$	Primary monocyte	12.0
SIV_{mac251}	MT-4 lymphoid	100.0

HIV strains: IIIb, MN, and RFII, T-lymphoid-cell-adapted variants; 112, 139-8, 421, 5002, 5003, and 5004, primary isolates; 139-8, highly resistant to several nonnucleoside RT inhibitors; 421, resistant to AZT; SF162, a monocytotropic variant. The 95% inhibitory concentration (IC_{95}) was defined as the concentration of test compound that inhibited virus growth by at least 95% compared to untreated infected control cultures. Reported values are representative of multiple determinations.

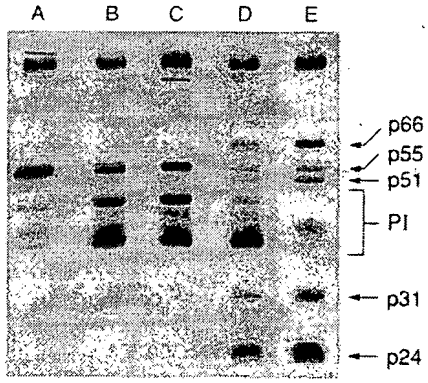

FIG. 2. Inhibition of HIV-1 precursor polyprotein processing by L-735,524 and effect on processing of inhibitor removal. Viral particles were analyzed immediately after harvest from inhibitor-treated cells (lanes A and B) or 6 h after removal of the inhibitor by dialysis (lanes C and D). L-735,524 was used at concentrations of 12.0 μM (lanes A and C) or 0.5 μM (lanes B and D). Lane E is mature virus from untreated cells. p24 is the processed viral capsid protein, p55 is the core precursor polyprotein, PI represents the core processing intermediates, p66 and p51 are the processed RT subunits, and p31 is the mature processed viral integrase enzyme.

Effect of Inhibitor Removal on Mature Virion Production. Persistently infected human T-lymphoid cells were treated with 0.5 μM L-735,524 for 48 h as described for the core protein processing experiment. The cells were then washed extensively, and the subsequent production of infectious virus was assayed by viral plaque titration (21). The amount of infectious virus produced in the 60 min immediately after removal of the inhibitor was identical to that produced in the same time interval by an untreated control culture (data not shown).

In a second study, immature virus particles produced by persistently infected cells treated with L-735,524 were harvested, and the inhibitor was removed from the culture medium by large-volume dialysis. After 6 h of continued incubation in the absence (<50 nM) of the compound, the viral particles were concentrated and analyzed by gel electrophoresis. An increased quantity of mature viral proteins and processing intermediates was noted relative to particles analyzed immediately after harvesting (Fig. 2). However, this apparent increase in protease activity after removal of L-735,524 was not accompanied by an increase in viral infectivity, as assessed by viral plaque assay (data not shown).

Mutant Protease Resistance. Numerous unsuccessful attempts have been made to derive L-735,524-resistant HIV-1 in cell culture by passage of wild-type virus in increasing concentrations of the inhibitor (unpublished observations). An effort was therefore undertaken to study potential resistance to the compound by constructing a series of mutant recombinant protease enzymes and assessing their susceptibility to L-735,524. The specific substitutions were chosen on the basis of modeling studies performed using the crystal structure of the HIV-1 protease complexed with a related inhibitor. The results (Table 2) showed that a number of conservative substitutions mediated a significant loss of susceptibility to the compound. However, in all cases this apparent resistance was accompanied by a notable decrease in the mutant enzyme's k_{cat}/K_m. Such mutations may, therefore, be detrimental to virus growth and may not be readily selected in cell culture.

Pharmacokinetic Profile. The pharmacokinetic profile of L-735,524 in rats, dogs, and monkeys is summarized in Table 3. After i.v. administration (5.0 mg/kg), L-735,524 was cleared very rapidly in rats. The large volume of distribution

Table 2. Inhibition by L-735,524 of mutant HIV-1 protease

Protease	K_i, nM	k_{cat}/K_m, sec^{-1}-mM^{-1}
Wild type (HIV-1)	0.358 ± 0.034	97.07 ± 8.70
Wild type (HIV-2)	3.316 ± 0.300	0.42 ± 0.14*
L23I	0.585 ± 0.042	26.67 ± 4.28
L23V	2.812 ± 0.326	4.70 ± 0.28
V32I	2.638 ± 0.249	4.22 ± 1.02*
I47V	0.767 ± 0.158	12.04 ± 3.77*
I47L	6.615 ± 0.495	0.10 ± 0.01
I50L	0.323 ± 0.070	2.57 ± 0.12
L76M	0.114 ± 0.017	54.95 ± 5.03*
V82I	0.052 ± 0.008	138.24 ± 21.53*
I84L	0.227 ± 0.018	11.65 ± 0.15
I84V	3.107 ± 0.393	3.16 ± 0.07

L-735,524 acted as a competitive inhibitor of all the mutant enzymes. Values were derived from two or more experiments. Each mutant enzyme expressed the noted amino acid substitution at the indicated protease residue position.
*These values were reported previously in Sardana *et al.* (19) and are included here for comparison.

(approximately three times that of body water) indicated extensive tissue binding of the drug. In spite of this, it had a relatively short half-life in plasma because of its high clearance rate. When L-735,524 was given to rats orally (20.0 mg/kg) as a solution in 0.05 M citric acid, the drug was fairly well absorbed. The plasma concentration peaked at about 2.8 μM in 0.5 h, and bioavailability (calculated by comparison between the i.v. and oral profiles) was 23%. Essentially identical plasma profiles were observed in rats when the drug was administered as a suspension in 0.5% methylcellulose (data not shown).

L-735,524 was also absorbed well after oral administration to dogs as a solution in citric acid (Fig. 3), and the oral bioavailability was estimated to be 70%. When delivered as a suspension in 0.5% methylcellulose, absorption was variable and notably less than when given as a solution (data not shown). However, oral bioavailability was still significant at 10–36%.

Oral administration of an L-735,524-containing solution to monkeys resulted in large individual variability. Nonetheless, bioavailability was estimated to be ≈14%.

Plasma Protein Binding. L-735,524 was not highly bound to rat, dog, or human plasma. The unbound fraction for this compound in plasma was 38%, 15%, and 56%, respectively.

DISCUSSION

Biochemical and genetic studies have established the HIV-1 protease as a significant target for antiviral therapeutic agents (4–6). As a result, many potent and selective inhibitors of the enzyme, based upon the transition-state mimic concept, have been reported. These compounds are peptidomimetics, and few have been tested in humans because of a lack of adequate oral bioavailability in animal models. One exception is Ro 31-8959 (compound 1) (22), which is a potent HIV protease inhibitor currently in phase II clinical trials. This compound

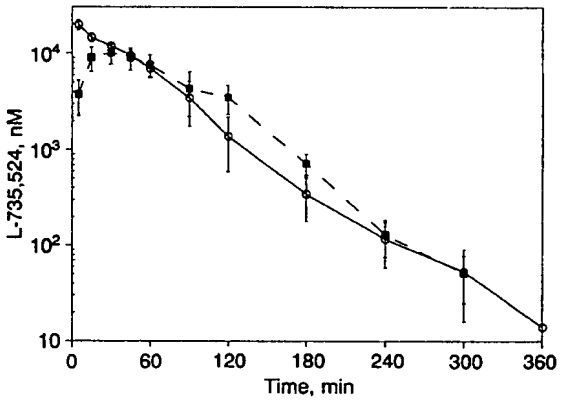

FIG. 3. Plasma concentration of L-735,524 in dogs (n = 4) after either intravenous (○) or oral (■) administration of a dose of 10 mg/kg.

belongs to the hydroxyethylamine class of inhibitors and contains a basic amine in its backbone that imparts modest aqueous solubility. Recently, both L-685,434 (compound 2) and L-689,502 (compound 3) have been disclosed (12, 13) as potent inhibitors of HIV-1 protease. Despite their non-amino acid structure, both compounds possess little oral bioavailability in animal models, possibly due to their poor aqueous solubility. These characteristics severely restrict their potential use for chronic therapy.

L-704,486 (compound 4) represents a series of HIV-1 protease inhibitors designed to exhibit increased aqueous solubility while maintaining acceptable levels of activity. This compound incorporates the basic amine found in Ro 31-8959 (compound 1) into the backbone of hydroxyethylene isosteres 2 and 3. Further modification of the basic amine group in compound 4 led to the optimum inhibitor L-735,524. L-735,524 was notably more soluble in water than compound 3 or 4, particularly at acidic pH, and its cell culture anti-HIV-1 activity was comparable to that reported for structurally different inhibitors that have proceeded to clinical evaluation (22, 23).

The compound was equivalently active against T-lymphoid-cell-adapted variants of HIV-1 and primary virus isolates. It also inhibited virus variants resistant to AZT, a clinically used nucleoside analog inhibitor of the viral RT enzyme, and to nonnucleoside RT inhibitors. L-735,524 employed in combination with either nucleoside analogs or nonnucleoside RT inhibitors displayed synergistic antiviral activity, suggesting that the inhibitor may be beneficially used in combination clinical therapy.

The repression of virus replication in cell culture correlated with L-735,524-mediated inhibition of viral precursor polyprotein processing. Removal of the compound from chronically infected cells resulted in immediate resumption of infectious virion production. In contrast, and of possible importance for practical clinical application, removal of the inhibitor from unprocessed noninfectious particles was fol-

Table 3. Pharmacokinetic profile of L-735,524 in animals

	Intravenous administration			Oral administration		
Animal	Cl, ml per min per kg	Vdss, liter/kg	$t_{1/2}$, min	C_{max}, μm	T_{max}, min	F%
Rat	79 ± 18	2.17 ± 0.84	28.5 ± 2.2	2.80 ± 1.05	30 ± 0.0	22.9
Dog	16 ± 2.1	0.725 ± 0.08	34.5 ± 5.5	11.4 ± 2.3	30 ± 0.0	71.6 ± 13.4
Monkey	26.6 ± 0.7	2.25 ± 0.62	74.3 ± 33.7	0.71 ± 0.24	65 ± 23	14.2 ± 2.8

L-735,524 was delivered orally in 0.05 M citric acid. F% was determined by comparing the mean areas under the curves after i.v. and oral doses. Cl, plasma clearance rate; Vdss, volume of distribution; $t_{1/2}$, plasma half-life; C_{max}, maximum plasma concentration; T_{max}, time of maximum plasma concentration; F%, percent orally bioavailable. Three or four animals were used per group. Data are the mean ± SEM.

lowed by some proteolytic processing, but this was apparently insufficient to yield infectious particles.

The *in vivo* selection of viral variants resistant to the RT inhibitors has limited or abrogated the usefulness of these compounds for long-term therapy of HIV-1 infection (24, 25). The cell culture isolation of viral variants with reduced susceptibility to HIV-1 protease inhibitors has been reported (26, 27). Accordingly, a significant effort was made to derive a variant resistant to L-735,524. However, after numerous cell culture selection attempts, using both lymphoid cell-adapted virus and primary viral isolates from humans, such a variant could not be isolated. This differs from our previous efforts to derive resistant viral variants to the nonnucleoside RT inhibitors. Such variants, expressing single nucleotide mutations, were readily isolated after only several cell culture passages of the wild-type virus in the presence of the inhibitor (27). This implies that virus expressing reduced susceptibility to L-735,524 is poorly represented in the wild-type virus population. This may be due to a hypothetical requirement for simultaneous expression of multiple mutations or to a severely diminished relative replicative capacity. In fact, we observed that the introduction of amino acid substitutions into recombinantly expressed HIV-1 protease that reduced the enzyme's susceptibility to L-735,524 also reduced the enzyme's k_{cat}/K_m. This reduced activity may result in debilitated virus. However, the significance of viral resistance to the eventual usefulness of L-735,524 can only be determined by clinical studies.

The most important attribute of L-735,524 is its apparent oral bioavailability in animal models when given in clinically acceptable formulations. The compound's bioavailability when delivered as a citric acid solution or as a solid suspension ranged from 10% to 70%, depending on the individual animal and species. These values are significantly better than those we have observed with previously reported inhibitors (unpublished data). This characteristic, along with L-735,524's apparently low plasma protein binding, suggests that the compound can be effectively delivered to humans in a convenient oral formulation. Phase I human studies with such a formulation should assess the inhibitor's safety, pharmacokinetics, and *in vivo* antiviral activity.

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1. Ratner, L., Haseltine, W., Patarca, R., Livak, K. J., Starcich, B., Josephs, S. F., Doran, E. R., Rafalski, J. A., Whitehorn, E. A., Baumeister, K., Ivanoff, L., Petteway, S. R., Jr., Pearson, M. L., Lautenberger, J. A., Papas, T. S., Ghrayeb, J., Chang, N. T., Gallo, R. C. & Wong-Stall, F. (1985) *Nature (London)* **313**, 277–284.
2. Toh, H., Ono, M., Saigo, K. & Miyata, T. (1985) *Nature (London)* **315**, 691.
3. Pearl, L. H. & Taylor, W. R. (1987) *Nature (London)* **329**, 351–354.
4. Seelmeier, S., Schmidt, H., Turk, V. & von der Helm, K. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 6612–6616.
5. Mous, J., Heimer, E. P. & LeGrice, S. F. (1988) *J. Virol.* **62**, 1433–1436.
6. Kohl, N. E., Emini, E. A., Schleif, W. A., Davis, L. J., Heimbach, J. C., Dixon, R. A. F., Scolnick, E. M. & Sigal, I. S. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 4686–4690.
7. Johnston, M. I., Alluadeen, H. S. & Sarver, N. (1989) *Trends Pharmacol. Sci.* **10**, 305–307.
8. Dunn, B. M. & Kay, J. (1990) *Antiviral Chem. Chemother.* **3**, 3–8.
9. Huff, J. R. (1991) *J. Med. Chem.* **34**, 2305–2314.
10. Tomasselli, A. G., Howe, W. J., Sawyer, T. K., Wlodawer, A. & Heinrikson, R. L. (1991) *Chim. Oggi*, 6–27.
11. Trova, M. P., Babine, R. E., Byrn, R. A., Casscles, W. T., Hastings, R. C., Hsu, G. C., Jirousek, M. R., Johnson, B. D., Kerwar, S. S., Schow, S. R., Wissner, A., Zhang, N. & Wick, M. M. (1993) *Biomed. Chem. Lett.* **3**, 1681–1686.
12. Lyle, T. A., Wiscount, C. M., Guare, J. P., Thompson, W. J., Anderson, P. S., Darke, P. L., Zugay, J. A., Emini, E. A., Schleif, W. A., Quintero, J. C., Dixon, R. A. F., Sigal, I. S. & Huff, J. R. (1991) *J. Med. Chem.* **34**, 1228–1230.
13. Thompson, W. J., Fitzgerald, P. M. D., Holloway, M. K., Emini, E. A., Darke, P. L., McKeever, B. M., Schleif, W. A., Quintero, J. C., Zugay, J. A., Tucker, T. J., Schwering, J. E., Homnick, C., Nunberg, J., Springer, J. P. & Huff, J. R. (1992) *J. Med. Chem.* **35**, 1685–1701.
14. Young, S. D., Payne, L. S., Thompson, W. J., Gaffin, N., Lyle, T. A., Britcher, S. F., Graham, S. L., Scholtz, T. H., Deana, A. A., Darke, P. L., Zugay, J., Schleif, W. A., Quintero, J. C., Emini, E. A., Anderson, P. S. & Huff, J. R. (1992) *J. Med. Chem.* **35**, 1702–1709.
15. Askin, D. A., Eng, K. K., Rossen, K., Purick, R. M., Wells, K. M., Volante, R. P. & Reider, P. J. (1994) *Tetrahedron Lett.* **35**, 673–676.
16. Heimbach, J. C., Garsky, V. M., Michelson, S. R., Dixon, R. A. F., Sigal, I. S. & Darke, P. L. (1989) *Biochem. Biophys. Res. Commun.* **164**, 955–960.
17. Anson, M. L. (1938) *J. Gen. Physiol.* **22**, 79.
18. Elion, G. B., Sivger, S. & Hitchings, G. B. (1954) *J. Biol. Chem.* **208**, 477–488.
19. Sardana, V., Schlabach, A. J., Graham, P., Bush, B. L., Condra, J. H., Culberson, C., Gotlib, L., Graham, D. J., Kohl, N. E., LaFemina, R. L., Schneider, C. L., Wolanski, B. S., Wolfgang, J. & Emini, E. A. (1994) *Biochemistry* (1994) **33**, 2004–2010.
20. Williams, J. W. & Morrison, J. F. (1979) *Methods Enzymol.* **63**, 437–467.
21. Harada, S., Koyanagi, Y. & Yamamoto, N. (1985) *Science* **229**, 563–566.
22. Roberts, N. A., Martin, J. A., Kirchington, D., Broadhurst, A. V., Craig, J. C., Duncan, I. B., Galpin, S. A., Handa, B. K., Kay, J., Krohn, A., Lambert, R. W., Merrett, J. H., Mills, J. S., Parkes, K. E. B., Redshaw, S., Ritchie, A. J., Taylor, D. L., Thomas, G. J. & Machin, P. S. (1990) *Science* **248**, 358–361.
23. Kempf, D. J., Marsh, K. C., Paul, D. A., Knigge, M. F., Norbeck, D. W., Kohlbrenner, W. E., Codacovi, L., Vasavanonda, S., Bryant, P., Wang, X. C., Wideburg, N. E., Clement, J. J., Plattner, J. J. & Erickson, J. (1991) *Antimicrob. Agents Chemother.* **35**, 2209–2214.
24. Larder, B. A., Darby, G. & Richman, D. D. (1989) *Science* **243**, 1731–1734.
25. Saag, M. S., Emini, E. A., Laskin, O. L., Douglas, J., Capidus, W. I., Schleif, W. A., Whitley, R. J., Byrnes, V. W., Hildebrand, C., Kappes, J. C., Anderson, K. W., Massari, F. E., Shaw, G. M. & the L-697,661 Working Group (1993) *N. Engl. J. Med.* **329**, 1065–1072.
26. Otto, M. J., Garber, S., Winslow, D. L., Reid, C. D., Aldrich, P., Jadhav, P. K., Patterson, C. E., Hodge, C. N. & Cheng, Y.-S. E. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 7543–7547.
27. Nunberg, J. H., Schleif, W. A., Boots, E. J., O'Brien, J. A., Quintero, J. C., Hoffman, J. M., Emini, E. A. & Goldman, M. E. (1991) *J. Virol.* **65**, 4887–4892.